IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

re Application of)
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ANDREW J. DANNENBERG)
) Appeal No. 2003-1935
Patent Application No. 09/554,604)
)
Filed: May 31, 2000)
)
For: CYCLOOXYGENASE-2 INHIBITION)

SUBMISSION OF PUBLICATION
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(In Triplicate)

Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith for consideration in the appeal is a copy of Yamamato, H., et al,

Gastroenterology 125, 556-571 (2003).

Good and sufficient reason why the Yamamoto et al article was not presented earlier, is

that the article first published in July 2003.

We turn now to the relevance of Yamamoto et al.

The claims are rejected under 35 U.S.C. 103 as obvious over Gregory et al U.S. Patent

No. 6,172,096 in view of Talley et al U.S. Patent No. 5,643,933 on the basis that Gregory et al

and Talley et al indicate selective inhibitors of COX-2 are known to have anti-inflammatory effect

and that Gregory et al suggests (no data is presented on this in Gregory) that selective inhibitors

of COX-2 are useful to treat biliary cirrhosis and prevent or delay liver transplant rejection.

One issue involved in the appeal is whether the combination of Gregory et al and Talley et al provides a reasonable expectation of success for benefit from *in vivo* treatment of chronic hepatitis B, chronic hepatitis C, alcoholic liver injury and nonalcoholic steatohepatitis by administration of a selective inhibitor of COX-2.

Evidence has already been presented supporting the conclusion of no expectation of success.

The enclosed article is submitted to constitute additional evidence supporting the conclusion of no expectation of success.

This additional evidence is recitation in the publication suggesting that the utility of selective inhibiting of COX-2 is unobvious and requires testing to establish, even when the same utility is known for nonselective COX inhibitors (inhibit COX-1 and COX-2); see first paragraph under discussion at page 564 of Yamamoto et al. Thus, Yamamoto et al had to carry out testing for a selective inhibitor of COX-2 to determine utility even though nonselective COX inhibitors has previously been found useful for the same purpose.

Please note that Gastroenterology is the leading publication in the field and it publishes only non-obvious results of importance and not pedestrian predictions. The fact that Gastroenterology published the article, is evidence of the non-obviousness of the results.
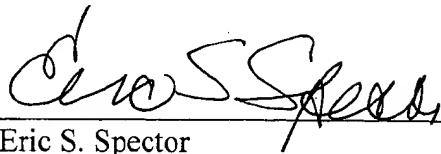
Application of the above here indicates that those skilled in the field would not have a reasonable expectation of success for the claimed method, without some test to support the result. Note that the instant case is even more persuasive of no expectation of success in respect to treatment of hepatitis C, because while in Yamamoto's case non-selective COX inhibitors did

work for its purpose, non-selective inhibitors of COX (NSAIDs) have been shown not to provide benefit in respect to treating hepatitis C (see page 10 of applicant's Brief).

Reversal of the rejection is requested.

Respectfully submitted,

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JTE-522, a Cyclooxygenase-2 Inhibitor, Is an Effective Chemopreventive Agent Against Rat Experimental Liver Fibrosis

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Background & Aims: The aim of this study was to assess the effects of cyclooxygenase (COX)-2 inhibition on rat experimental liver fibrogenesis. *Methods:* We investigated the inhibitory effects of a selective COX-2 inhibitor, JTE-522, on liver fibrosis induced by a choline-deficient, L-amino acid—defined diet (CDAA). Inhibitory effect was also tested in a second model of thioacetamide (TAA)-induced liver fibrosis. *Results:* CDAA induced liver fibrosis and preneoplastic foci at 12 weeks and cirrhosis at 36 weeks. Hepatocellular carcinoma was noted in 13 of 15 rats (87%). JTE-522 significantly inhibited fibrosis and development of preneoplastic lesions in a dose-dependent manner and completely inhibited generation of cirrhosis and hepatocellular carcinoma at both low and high doses (10 and 30 mg/kg body wt/day, respectively). JTE-522 administrated only from 12 weeks to 36 weeks also prevented cirrhosis and formation of hepatocellular carcinoma. JTE-522 itself did not cause local or systemic gross or histopathologic changes at 36 weeks. Mechanistic studies indicated that the CDAA model displayed up-regulation of several biomarkers, including COX-2, arachidonate metabolite (prostaglandin E_2), serum aspartate aminotransferase, and c-myc expression. The model also showed an increased proportion of activated hepatic stellate cells, proliferating cell nuclear antigen index, and CD45-positive inflammatory cells in the liver. JTE-522 effectively diminished these changes. JTE-522 exhibited similar antifibrosis effects in the TAA model. *Conclusions:* Our results suggest that COX-2 is involved in CDAA- and TAA-induced liver fibrosis. Our data also indicate that JTE-522 is a potent chemopreventive agent of rat liver fibrosis with low toxicity.

Primary hepatocellular carcinoma (HCC) is one of the most common tumors in Southeast Asia and Africa. The prognosis of HCC is generally poor, and the 5-year survival rate is limited to 25%–49% after surgery. In the United States, it has been estimated that 400,000 people have cirrhotic liver disease or cirrhosis; 13,900 new cases of HCC were diagnosed in 1998.[1] Most patients diagnosed with HCC have underlying liver cirrhosis commonly caused by infection with hepatitis C virus. Similarly, hepatitis C virus–based liver cirrhosis is a serious problem in Japan because it develops to HCC at a rate of 5%–7% per year.[2] Therefore, an effective strategy to prevent cancer development is strongly desired. Because fibrosis is a prominent event that largely increases from hepatitis to cirrhosis, an effective strategy might be to prevent progression of liver fibrosis.

Cyclooxygenase (COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin (PG) H_2, the precursor of various compounds including PGs, prostacyclin, and thromboxanes. Two COX genes, COX-1 and COX-2, have been identified that share more than 60% identity at the amino acid level.[3] COX-1 is constitutively expressed in many tissues and is responsible for various physiologic functions, including cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanoid, thromboxane A_2, by platelets. On the other hand, COX-2 is an inducible immediate-early gene and was originally found to be induced by various stimuli, such as mitogens and cytokines, and growth factors.[4] Overexpression of COX-2 has been reported in various chronic inflammatory diseases.[5]

In recent years, we and other investigators have shown increased COX-2 expression in various types of human cancers and precursor lesions.[6–9] The relevance of COX-2 in human neoplasia has been best proven in patients with familial adenomatous polyposis. Double-knockout mice for the *APC* and *COX-2* genes showed marked reduction in the size and frequency of intestinal polyps, and studies using COX-2 inhibitors inhibited similar results in Apc knockout mice.[10,11] Finally, celecoxib, a selective COX-2 inhibitor, significantly inhibited growth and genesis of colon polyps in patients with familial adenomatous polyposis.[12]

In human hepatocarcinogenesis, the above scenario has not yet been completely delineated. We previously showed that COX-2 expression is up-regulated from normal liver to chronic hepatitis and the highest COX-2 expression was noted in liver cirrhosis, which was even higher than the levels in HCC.[8] Other investigators also showed a relatively high COX-2 expression in early HCC but not advanced HCC.[7] These findings suggest that COX-2 may participate in liver fibrogenesis rather than cancer progression. However, evidence on liver fibrosis or HCC in relation to COX-2 is limited, and the functional significance of COX-2 expression in the process of liver fibrosis has not yet been confirmed.

To explore the effects of COX-2 inhibition against liver fibrosis and cirrhosis as well as HCC, we conducted in vivo animal studies using a novel selective COX-2 inhibitor, JTE-522 (4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide), which at 100 μmol/L is known to selectively inhibit in-vitro COX-2 activity without affecting COX-1.[13,14] Previous studies have shown that JTE-522 inhibits polypogenesis in Apc knockout mice, hematogenous metastasis of colon cancer cells, and N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in various animal models.[15–17] To induce liver cirrhosis in rats, we used a choline-deficient, L-amino acid—defined diet (CDAA).[18,19] A choline-deficient diet is a primeval carcinogen for rat hepatocarcinogenesis, and CDAA is a choline-deficient modified diet (choline-deficient diet plus L-amino acid) that exerts more potent carcinogenicity. This model of liver cirrhosis displays 2 phenomena: (1) formation of liver fibrosis and preneoplastic foci at the early phase, and (2) development of cirrhosis and HCC at the late phase. To confirm the effects of JTE-522 noted in CDAA-fed rats, we also used a second model of rat liver fibrosis induced by thioacetamide (TAA).[20]

Mat rials and M thods

Animals, Diets, and COX-2 Selective Inhibitor

A total of 116 male 5-week-old Wistar rats weighing 150–153 g were purchased from Shionogi Co. (Osaka, Japan).

Table 1. Experimental Protocol

Experiment	n	Length of experiment (wk)
A		
CDAA alone	5	12
CDAA + JTE-522 (10 mg · kg⁻¹ · day⁻¹)	5	12
CDAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	5	12
CSAA alone	5	12
B		
CDAA alone	5	36
CDAA + JTE-522 (10 mg · kg⁻¹ · day⁻¹)	10	36
CDAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	10	36
CSAA alone	10	36
CSAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	10	36
C		
CDAA + JTE-522 (10 mg · kg⁻¹ · day⁻¹)	10	CDAA (12)/JTE-522 in CDAA (12–36)
CDAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	10	CDAA (12)/JTE-522 in CDAA (12–36)

They were housed in an air-conditioned room maintained at 25°C with a 12-hour dark/light cycle. The control diet (choline-supplemented, L–amino acid—defined diet [CSAA], no. 518754) and CDAA (no. 518753) were obtained from Dyets Inc. (Bethlehem, PA). The amino acid composition of the CDAA and CSAA was made up only of pure L–amino acids with the exception of glycine. For the TAA-induced liver fibrosis model, regular laboratory chow diet was used. The selective COX-2 inhibitor, JTE-522, was obtained from Japan Tobacco, Inc. (Tokyo, Japan).[13,14] JTE-522 was dissolved in ethanol and premixed with CDAA or chow diet once per month by Central Laboratories for Experimental Animals Inc. (Tokyo, Japan) and stocked at 4°C in the dark. Diets were replaced every week, and body weight was monitored weekly.

Experimental Protocols

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

CDAA model. Table 1 summarizes the protocols for the CDAA model. Experiment A was designed as a 12-week protocol to examine the effects of JTE-522 on early pathologic stages, at which significant liver fibrosis and preneoplastic foci are known to appear.[18,19] This experiment included 4 groups of 5 rats each. Group 1 received CDAA alone, groups 2 and 3 received CDAA containing JTE-522 at a dose of either 10 mg · kg⁻¹ · day⁻¹ or 30 mg · kg⁻¹ · day⁻¹, respectively, and group 4 received the control diet (CSAA alone). Experiment B, a 36-week protocol, was designed based on the report that HCC develops after 32 weeks[18,19] to examine the effects of JTE-522 on late events such as formation of

cirrhosis. Rats were divided into 5 groups consisting of 15 or 10 rats each. In groups 1–4, the diet contents were the same as those in experiment A. Group 5 (CSAA plus JTE-522 at 30 mg · kg^{-1} · day^{-1}) was set to examine the potential toxicity of JTE-522. Experiment C was designed to examine the effects of delayed administration of JTE-522. Thus, groups 1 and 2 received CDAA alone during the initial 12 weeks and then CDAA plus JTE-522 at a low or high dose for the subsequent 24 weeks (12–36 weeks).

TAA-induced liver fibrosis model. Control groups (n = 5) were given chow diet for 6 weeks. Liver fibrosis was produced by repeated intraperitoneal injections of TAA (200 mg/kg; Wako Pure Chemical Industries, Osaka, Japan) 3 times per week for 6 weeks (n = 8).[20] In another group, 8 rats that received TAA injection were fed chow diet plus JTE-522 at 15 mg · kg^{-1} · day^{-1} for 6 weeks.

The rats in each group were killed under light ether anesthesia at the end of each experiment. The livers and other organs were immediately dissected out and examined macroscopically. For histopathologic and immuno-histochemical examinations, tissue samples were fixed in 10% neutral buffered formalin, processed through graded ethanol solutions, and embedded in paraffin. A portion of the liver samples was immediately frozen in liquid nitrogen and stored at −80°C. Serum was collected from blood samples and stored at −80°C.

Reagents

Anti-murine COX-2 polyclonal antibody and the blocking peptide that was used to generate COX-2 antibody, the COX-2 electrophoresis standard, were obtained from Cayman Chemical (Ann Arbor, MI).[21] Anti-rat glutathione S-transferase placental form (GST-P) polyclonal antibody was obtained from Medical & Biological Laboratories Co. (Nagoya, Japan).[22] Anti-human α–smooth muscle actin (α-SMA) monoclonal antibody (clone 1A4), which cross-reacts with rat α-SMA but not mouse α-SMA, was obtained from Dako (Carpinteria, CA).[23] Anti-rat CD45 (also designated as leuko-cyte common antigen) monoclonal antibody was obtained from PharMingen (San Diego, CA).[24] Anti-human actin polyclonal antibody, which cross-reacts with rat actin, was obtained from Sigma Chemical Co. (St. Louis, MO). Anti-human monoclonal proliferating cell nuclear antigen (PCNA) antibody, which cross-reacts with rat PCNA, was purchased from Novocastra Laboratories (Benton Lane, Newcastle, England).[25]

Collagen Staining

Collagen staining was performed using a collagen staining kit (Collagen Research Center, Tokyo, Japan) according to the method described by the supplier.[26] Briefly, paraffin-embedded liver sections were prepared in 4-μm thickness, deparaffinized in xylene, and rehydrated. After washing twice with phosphate-buffered saline for 10 minutes each, the slides were incubated for 1 hour at room temperature with staining solution A, which specifically reacts with collagen. The slides were then washed 3 times with phosphate-buffered saline and once with distilled water for 5 minutes each. The tissue fraction containing collagen is stained in red.

Immunohistochemistry

Immunostaining was performed as described previously by our laboratories.[8,9] Briefly, after deparaffinization, heat antigen retrieval was performed in 10 mmol/L citrate buffer, pH 6.0, at 95°C for 40 minutes. The slides were then processed for immunohistochemistry on the TeckMate Horizon automated staining system (DAKO, Glostrup, Denmark) using the Vectastain ABC-peroxidase kit (Vector Laboratories, Burlingame, CA). In the step of primary antibody reaction, the slides were incubated with the GST-P, COX-2, CD45, and PCNA antibodies (final concentration: 1:500, 1:100, 1:100, and 1:50, respectively) for 1 hour at room temperature. For positive controls, Jurkat cells were used for CD45 staining[24] and human colon cancer tissue was used for COX-2 staining.[9] Normal crypts in the colon of rats served as a positive control for PCNA staining.[25] The control sections of GST-P–positive minifoci in the early stages of rat hepatocarcinogenesis were kindly provided by Medical & Biological Laboratories Co. The specificity of positive staining with COX-2 antibody was further verified by absorption test using blocking peptide. For negative controls, phosphate-buffered saline was used as a substitute for the primary antibody to exclude false-positive responses from nonspecific binding of immunoglobulin G or from the secondary antibody.

Evaluation of Histology, Liver Fibrogenesis, and GST-P–Positive Preneoplastic Nodules

The processes of liver fibrosis, cirrhosis, and HCC were assessed by H&E staining (by H.Y. and K.W.). The criteria of Squire and Levitt[27] were used for the diagnosis of rat HCC. A 30-cm^2 area, which corresponded to approximately 40–45 slides, was examined in each liver. For assessment of liver fibrogenesis, collagen-stained areas were measured by a computer-assisted image analysis system with Mac Scope software (Mitani Co., Fukui, Japan),[9] and the relative area of fibrosis to the whole liver was calculated. GST-P staining is a reliable marker of preneoplastic lesions in rat hepatocarcinogenesis.[22] The numbers of GST-P–positive preneoplastic nodules were counted under the microscope. The area of GST-P–positive preneoplastic nodules was measured by a computer-assisted image analysis system and expressed as positive area per 1 cm^2.

Evaluation of CD45 and PCNA Staining

For analysis of CD45 or PCNA index, the fields of periportal area or fibrotic septa of each liver sample were surveyed for CD45- or PCNA-positive cells. A 30-cm^2 area was examined in each liver, and positive cell number/cm^2 was calculated.

Western Blot Analysis

Approximately 50 mg of liver sample was homogenized in 0.5 mL of RIPA buffer (25 mmol/L Tris, pH 7.4, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, and 0.2% sodium dodecyl sulfate) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). The homogenate was centrifuged at 14,000 rpm for 20 minutes at 4°C. The resulting supernatant was collected, and total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Western blot analysis was performed as described in our previous studies.[28,29] Briefly, 100 μg of the total protein was premixed with loading buffer (0.05 mol/L Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 0.2 mol/L β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue), boiled for 5 minutes, and subjected to sodium dodecyl sulfate/polyacrylamide protein gel electrophoresis on 10% gel. After electrophoresis, protein transfer was performed onto a polyvinylidene difluoride membrane using a transblot apparatus in a buffer containing 0.02 mol/L Tris-HCl (pH 8.3), 0.2 mol/L glycine, and 20% methanol. After blocking in 10% skim milk, the membrane was incubated with the primary antibody, anti–COX-2 (1:100 dilution), anti–α-SMA (1:200 dilution), or anti-actin (1:1000 dilution) for 1 hour at room temperature. After 3 washes each for 5 minutes with Tris-buffered saline (0.02 mol/L Tris-HCl [pH 7.5] and 0.1 mol/L NaCl) containing 0.2% Tween 20, the filter was incubated with the secondary antibody at a dilution of 1:2000. The protein bands were detected using the Amersham enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) according to the instructions provided by the manufacturer. For positive controls, the purified COX-2 protein (Cayman Chemical) and human cirrhotic liver tissues that cross-react with the currently used anti–α-SMA antibody[23] were used. An absorption test was performed to verify the specificity of the COX-2 antibody.

Measurement of PGE₂ Levels

To measure basal PGE₂ levels in the liver, approximately 50-mg frozen liver samples were homogenized on ice in 0.5 mL of 0.1 mol/L Tris-HCl buffer and vortexed thoroughly for 3 minutes. PGE₂ levels in supernatants were determined using an enzyme immunoassay kit for PGE₂ (Cayman Chemical) and a UV detector (Wako Pure Chemical Industries) using the instructions provided by the manufacturer.[17]

Biochemical Assay of Aspartate Aminotransferase

Aspartate aminotransferase activities in the serum samples were enzymatically analyzed using a selective chemistry analyzer (Arkray, Osaka, Japan).

Quantitative Real-Time Polymerase Chain Reaction

The frozen tissue specimens were crashed in TRIzol reagent (Life Technologies, Vienna, Austria). RNA extraction was performed according to the instructions provided by the supplier. Purified RNA was quantified and assessed for purity by UV spectrophotometry. Complementary DNA was generated from 1 μg RNA with avian myeloblastosis virus reverse transcriptase using the Reverse Transcription System (Promega, Madison, WI) according to the instructions provided by the supplier. Quantitative polymerase chain reaction (PCR) was performed using Light Cycler (Roche Diagnostics, Mannheim, Germany) as described previously.[9] The PCR primers used for detection of c-myc and β-actin were prepared as reported previously.[30,31] Briefly, 20 μL of PCR reaction components, with Light Cycler/Fast Start DNA Master SYBRGreen I (Roche Diagnostics), contained 0.25 μmol/L of each primer, 2 μmol/L MgCl₂, and 2 μL complementary DNA as a template. PCR conditions were set up as follows: one cycle of denaturing at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 62°C for 10 seconds, and 72°C for 40 seconds. Fluorescence was acquired at the end of every 72°C extension phase. Quantification data from each sample were analyzed using Light Cycler analysis software. In this analysis, the background fluorescence was subtracted by setting a noise band. The concentration of the target was obtained by plotting against a standard curve. Rat IEC18 intestinal cell line was used as the standard (i.e., use of known concentrations). The amount of c-myc was normalized by that of β-actin. For verifying the specific product, we analyzed the melting temperature curves of final PCR products.

Statistical Analysis

Statistical analysis was performed using the Statview J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). For multiple comparisons, the Dunnett method was used together with least squares means to provide appropriate correction. Differences between 2 groups were analyzed by Student t test. $P < 0.05$ indicated a statistically significant difference.

Results

CDAA Model

Food intake. The amount of food intake of each type of diet was estimated every week up to 36 weeks and per rat for each group in experiment B, and no significant differences were found among the groups with regard to the consumed amount of different diets (CDAA and CSAA, with or without JTE-522, data not shown).

Results of experiment A (12 weeks). *Histopathologic changes.* Rats fed CDAA exclusively had several prominent histopathologic changes in the liver at 12 weeks, such as accumulation of free fatty acid in hepatocytes, increase in the number of fibrous septa, and loss of structure of hepatic lobules (Figure 1*A*). Addition of

Table 2. Results of Experiment A

Groups	No. of rats	Final body weight (g)[a]	Fibrosis (cm²/cm²)[a]	GST-P–positive lesions[a] No./cm²	GST-P–positive lesions[a] Area (mm²/cm²)
Experiment A					
CDAA alone	5	321 ± 3.0	0.132 ± 0.009	4.1 ± 0.5	0.22 ± 0.04
CDAA + JTE-522 (10 mg · kg⁻¹ · day⁻¹)	5	338 ± 4.9[b]	0.041 ± 0.002[c]	1.7 ± 0.2[d]	0.07 ± 0.03[c]
CDAA + JTE-522 (30 mg · kg⁻¹ · kg · day⁻¹)	5	345 ± 4.8[d,e]	0.007 ± 0.002[f,g]	0[c,g]	0[c,g]
CSAA alone	5	346 ± 2.5[f]	0.006 ± 0.001[f]	0[f]	0[f]

[a]Values are means ±SD.
[b]NS compared with group 1.
[c]P < 0.01, compared with group 1.
[d]P < 0.05, compared with group 1.
[e]P < 0.01, compared with group 2.
[f]P < 0.001, compared with group 1.
[g]P < 0.001, compared with group 2.

JTE-522 to CDAA inhibited these changes, and such inhibition was more evident at a higher dose than at a lower dose. Thus, the use of a low dose (10 mg · kg⁻¹ · day⁻¹) of JTE-522 resulted in reduction of fat accumulation and mild fibrosis (Figure 1B); at 30 mg · kg⁻¹ · day⁻¹, JTE-522 resulted in marked inhibition of fat accumulation and fibrogenesis with preservation of the hepatic architecture (Figure 1C). Figure 1D shows the histology of normal liver of rats fed the control CSAA.

Body weight, fibrosis, and GST-P–positive lesions. Table 2 summarizes the changes in body weight, extent of liver fibrosis, and number and area of GST-P–positive preneoplastic nodules. At 12 weeks, CDAA caused a decrease in body weight, which is a finding consistent with those of other reports.[18,19] CDAA caused extensive fibrosis, as detected by collagen staining (Figure 2A), and the appearance of GST-P–positive lesions (Figure 2B) in the livers compared with CSAA (Table 2). CSAA produced no GST-P–positive lesions at 12 weeks. Low-dose JTE-522 plus CDAA inhibited fibrogenesis and formation of GST-P–positive nodules. Furthermore, high-dose JTE-522 plus CDAA completely inhibited these changes and resulted in body weight similar to the

CSAA-treated control group, only minimal fibrosis, and no GST-P–positive nodules.

Results of experiment B (36 weeks). The gross appearances of representative livers in groups 1–4 of experiment B are shown in Figure 3. CDAA alone resulted in the development of fatty cirrhotic liver in all rats (Figure 3A), whereas other groups of rats did not show any gross changes (Figure 3B–D). Histopathologic examination showed that CDAA induced liver cirrhosis in 15 of 15 rats (100%) and HCC in 13 of 15 rats (87%) (Table 3 and Figure 4A). In contrast, no cirrhotic nodules or HCCs were noted in the other diet treatment groups. Rats fed CDAA and treated with low-dose JTE-522 showed only fatty changes and fibrogenesis in the liver (Figure 4B). However, rats fed CDAA and treated with high-dose JTE-522 showed only mild fatty changes and mild fibrogenesis (Figure 4C). No histopathologic changes were noted in the livers of the CSAA-alone group (Figure 4D). It was found that CDAA-treated groups exclusively showed marked infiltration of mononuclear cells into the fibrous tissues within the liver (Figure 4A), whereas JTE-522–treated groups showed only occasional infiltration.

Table 3. Incidence of Cirrhosis and HCC in Experiments B and C

Groups	Cirrhosis No. of rats	Cirrhosis Incidence (%)	HCC Incidence (%)	HCC No./cm²	HCC Area (mm²/cm²)
Experiment B					
CDAA alone	15	15 (100)	13 (87)	3.1 ± 0.9	0.43 ± 0.11
CDAA + JTE-522 (10 mg · kg⁻¹ · day⁻¹)	10	0 (0)	0 (0)	0	0
CDAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	10	0 (0)	0 (0)	0	0
CSAA alone	10	0 (0)	0 (0)	0	0
CSAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	10	0 (0)	0 (0)	0	0
Experiment C					
CDAA + JTE-522 (10 mg · kg⁻¹ · day⁻¹)	10	0 (0)	0 (0)	0	0
CDAA + JTE-522 (30 mg · kg⁻¹ · day⁻¹)	10	0 (0)	0 (0)	0	0
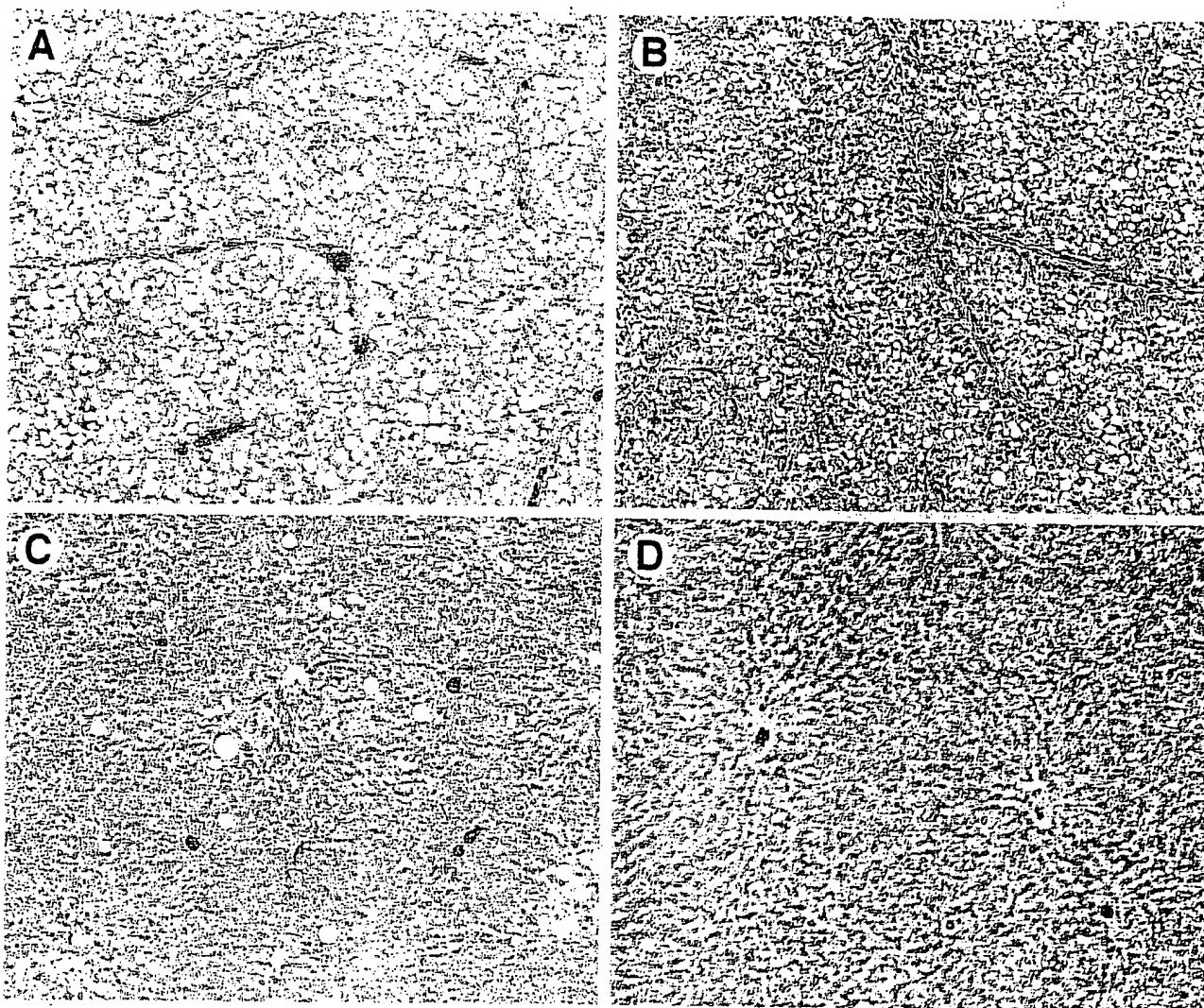
Figure 1. Histopathologic findings in livers of representative rats from each group in experiment A (12 weeks). Sections were stained with H&E. (*A*) CDAA caused marked fatty changes and fibrosis. CDAA plus JTE-522 at (*B*) 10 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} and (*C*) 30 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} inhibited histopathologic changes in a dose-dependent manner. (*D*) The liver of a representative rat fed control CSAA. (Original magnification: *A–D*, 100×.)
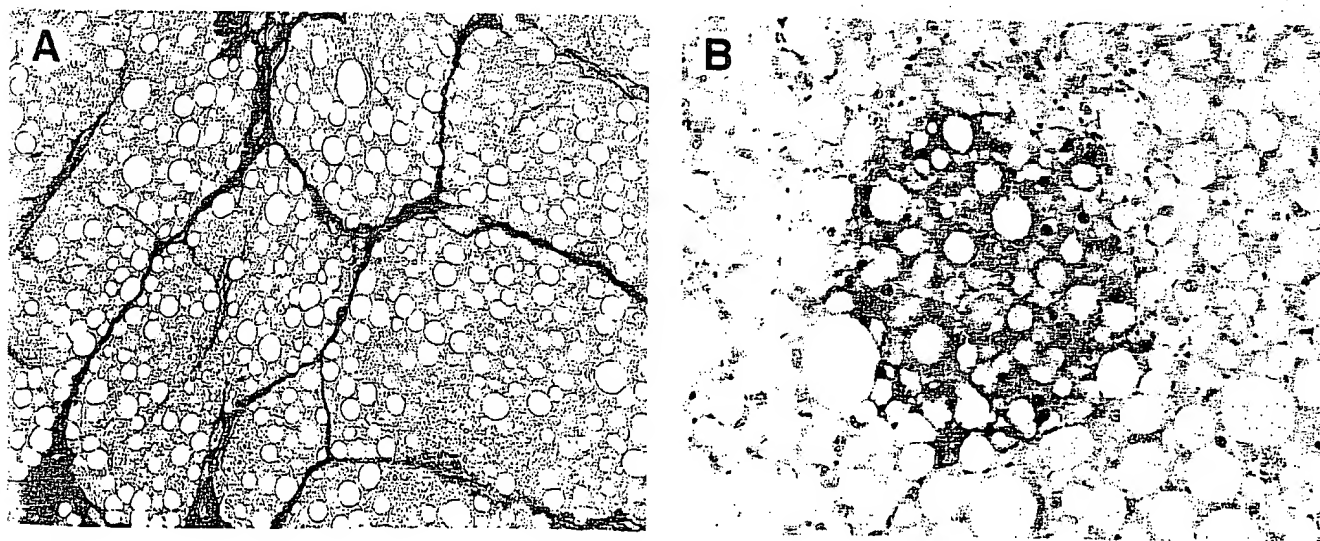
Figure 2. (*A*) Collagen staining and (*B*) staining of GST-P–positive preneoplastic nodule: (*A*) Collagen fibers are stained in red. (*B*) Representative GST-P–positive preneoplastic nodule in the liver of rats fed CDAA for 12 weeks. The control sections of GST-P–positive minifoci provided by Medical & Biological Laboratories Co. gave a similar staining pattern (data not shown). (Original magnification: *A*, 200×; *B*, 400×.)

Changes in body weight, fibrosis, and formation of GST-P–positive lesions in experiments B and C are shown in Figure 5. The values in experiment B were essentially an extension of those of experiment A. With CDAA treatment, the amount of fibrous tissue and the number of GST-P–positive nodules increased approximately 3 times and doubled, respectively, compared with those at 12 weeks. The area of GST-P–positive nodules was slightly increased. On the other hand, JTE-522 significantly inhibited CDAA-induced changes in a dose-dependent manner.

Toxicity of JTE-522. Group 5 in experiment B was set to monitor the toxicity of JTE-522. The body weights of rats fed CSAA alone or CSAA plus 30 mg · kg^{-1} · day^{-1} JTE-522 for 36 weeks were similar (Figure 5). Group 5 did not show any gross or histopathologic changes in the liver, kidneys, stomach (such as ulcer formation or hemorrhage), pancreas, bladder, intestine, brain, heart, or lungs (data not shown), suggesting that JTE-522 did not cause any apparent toxicity.

Results of experiment C. Administration of JTE-522 for 24 weeks had a lesser effect on inhibition of CDAA-induced fibrogenesis and formation of preneoplastic foci compared with the corresponding groups in experiment B (groups 2 and 3) that received continuous administration of JTE-522 for 36 weeks (Figure 5). No cirrhosis or HCC was noted in rats in experiment C (Table 3).

Expression of COX-2 in liver tissues. The COX-2 antibody yielded a strong band for the purified COX-2 protein, serving as positive control (Figure 6A, upper panel). COX-2 expression increased from normal liver to fatty liver with fibrosis to cirrhotic liver. JTE-522 at a high dose did not affect CSAA-associated low COX-2 expression. However, JTE-522 decreased CDAA-associated high COX-2 expression in a dose-dependent manner. For better comparison between the histopathologic changes and COX-2 expression, immunostaining for the COX-2 protein is shown in Figure 6B. In normal livers treated with CSAA for 36 weeks, COX-2 expression was undetectable (Fig. 6B, a). CDAA-induced fatty liver with fibrosis (12 weeks) expressed a moderate level of the COX-2 protein (Fig. 6B, b). Most cirrhotic livers in rats

treated with CDAA for 36 weeks showed marked COX-2 expression, mainly in the perinuclear region and cytoplasm of the hepatocytes. In addition, fibroblast-like cells and mononuclear cells in the fibrous septum also expressed COX-2 (Fig. 6B, c). In the groups treated with CSAA plus high-dose JTE-522 or CDAA plus high- or low-dose JTE-522 for 36 weeks (Fig. 6B, d–f), concordant results with Western blot were obtained.

Assessment of PGE_2 levels, aspartate aminotransferase levels, and PCNA expression. To examine arachidonate metabolism in the CDAA model, PGE_2 level in liver tissues was measured. CDAA led to an increase in PGE_2 level time-dependently compared with CSAA treatment, and JTE-522 inhibited the change in dose- and time-dependent manners (Figure 7A). When aspartate aminotransferase levels were examined by using serum samples, similar results were obtained (Figure 7B). PCNA expression was noted in the nuclei of hepatocytes (data not shown). PCNA index also exhibited similar results (Figure 7C).

CD45 index in liver tissues. CDAA induced CD45-positive inflammatory cells as early as 12 weeks, and few of these cells were noted at 36 weeks of CDAA treatment (Figure 8A, a and B). At 36 weeks, JTE-522 partially inhibited the appearance of such cells when used at a low dose (Figure 8A, b) and completely inhibited CD45-positive cells at a high dose (Figure 8A, c).

Expression of α-SMA in liver tissue. Because activated hepatic stellate cells play a role in liver fibrogenesis, we investigated whether JTE-522 affected the amount of activated hepatic stellate cells using α-SMA as a marker.[20,32] Western blot analysis indicated that α-SMA expression was increased from normal liver to fibrotic liver to cirrhosis and JTE-522 dose-dependently reduced α-SMA expression in the CDAA group (Figure 9).

Expression of c-myc messenger RNA. CDAA treatment for 36 weeks induced significantly higher expression of c-myc messenger RNA than CSAA treatment (Figure 10). Induction of c-myc gene expression was significantly inhibited by high-dose JTE-522 ($P <$ 0.01).

Figure 3. Macroscopic appearance of the livers of representative rats of experiment B. (*A*) CDAA alone resulted in the development of fatty cirrhotic liver, whereas (*B–D*) other groups did not show any gross changes (*B*, CDAA + JTE-522, 10 mg · kg^{-1} · day^{-1}; *C*, CDAA + JTE-522, 30 mg · kg^{-1} · day^{-1}; *D*, CSAA).

Figure 4. Histopathologic findings in livers of representative rats from each group in experiment B (36 weeks). Sections were stained with H&E. (*A*) CDAA-induced cirrhotic nodule (left) and HCC (right). Note the marked infiltration of mononuclear cells in the fibrous septa. (*B*) CDAA plus JTE-522 at 10 mg · kg^{-1} · day^{-1} resulted in massive fatty change and prominent fibrogenesis. Some inflammatory cells are present in the fibrous tissues (*arrows*). (*C*) CDAA plus JTE-522 at 30 mg · kg^{-1} · day^{-1} resulted in mild fatty changes and mild fibrogenesis. (*D*) Normal liver of a representative control CSAA rat. (Original magnification: *A–D*, 100×.)
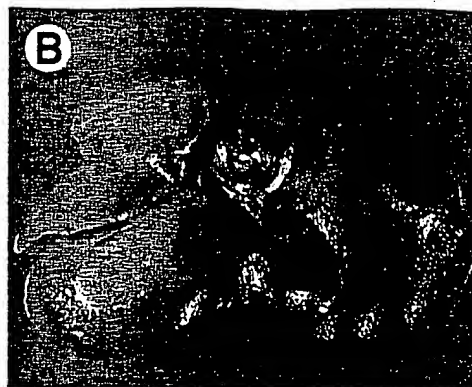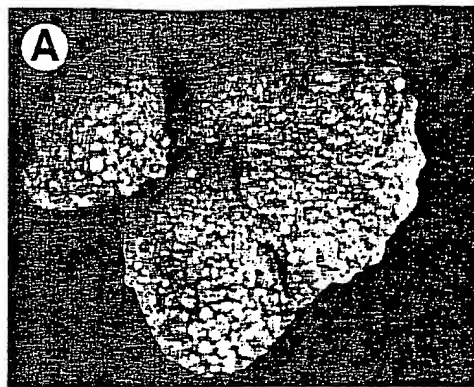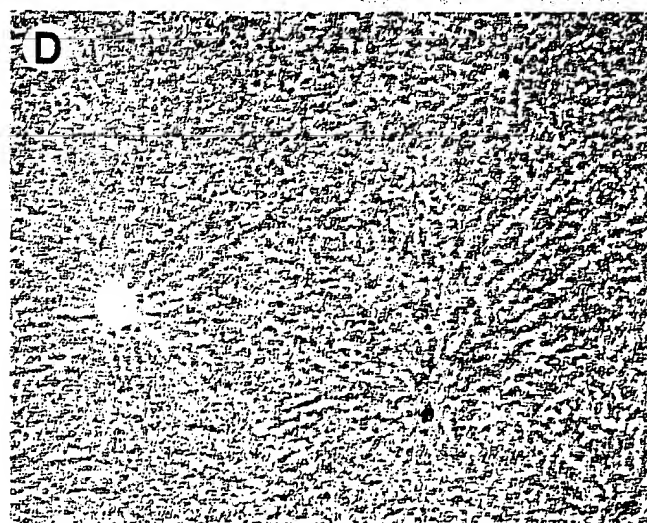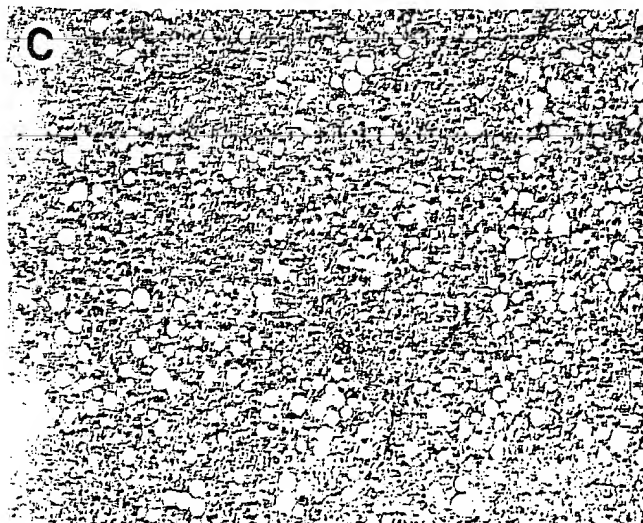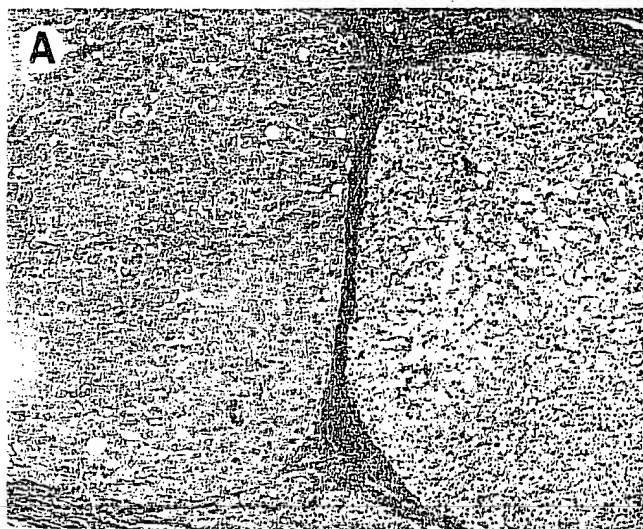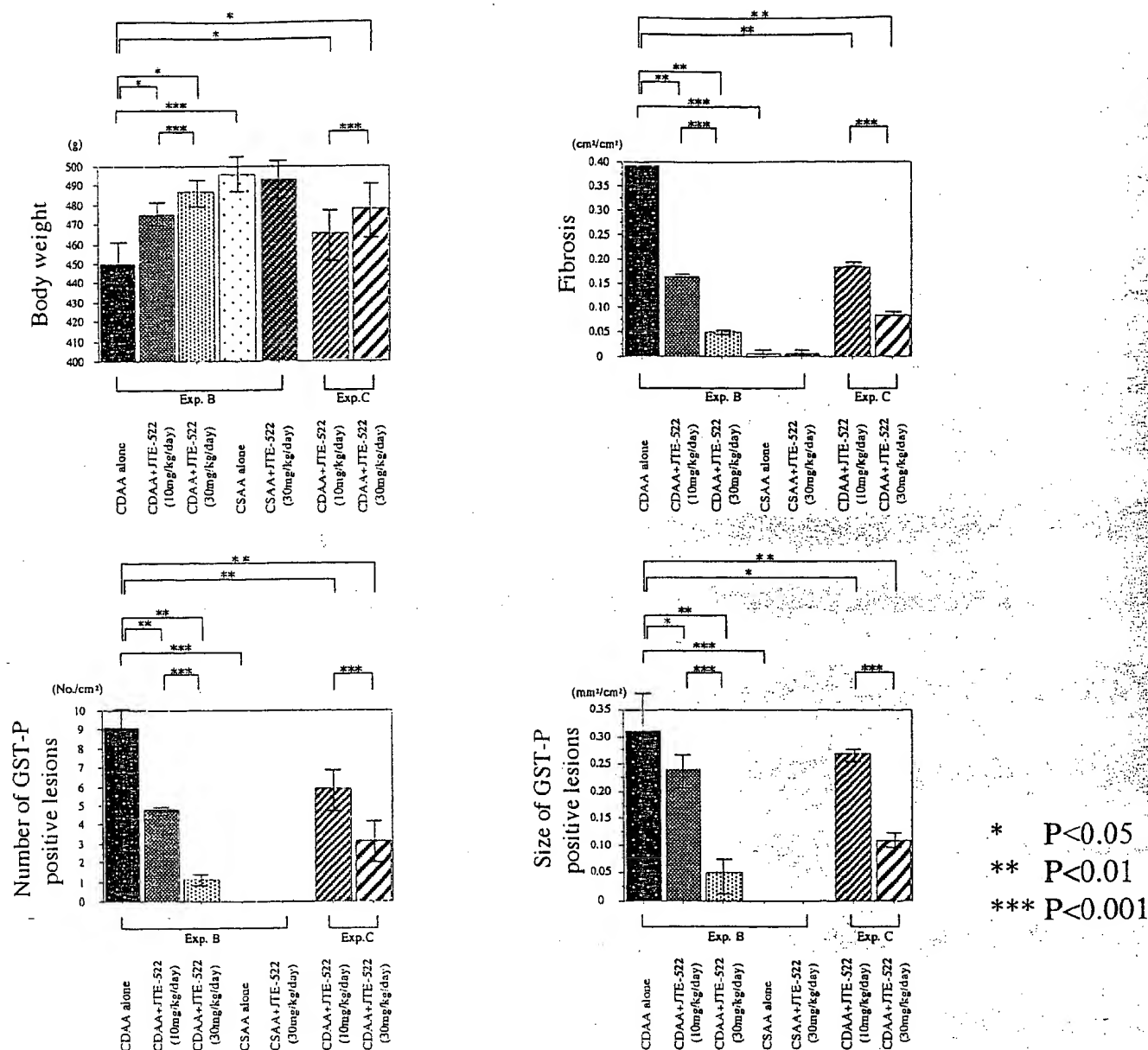
Figure 3.

Figure 4.

Figure 5. Quantitative analysis of changes in body weight, fibrosis, and GST-P–positive lesions in experiments B and C. Data are mean ± SD.
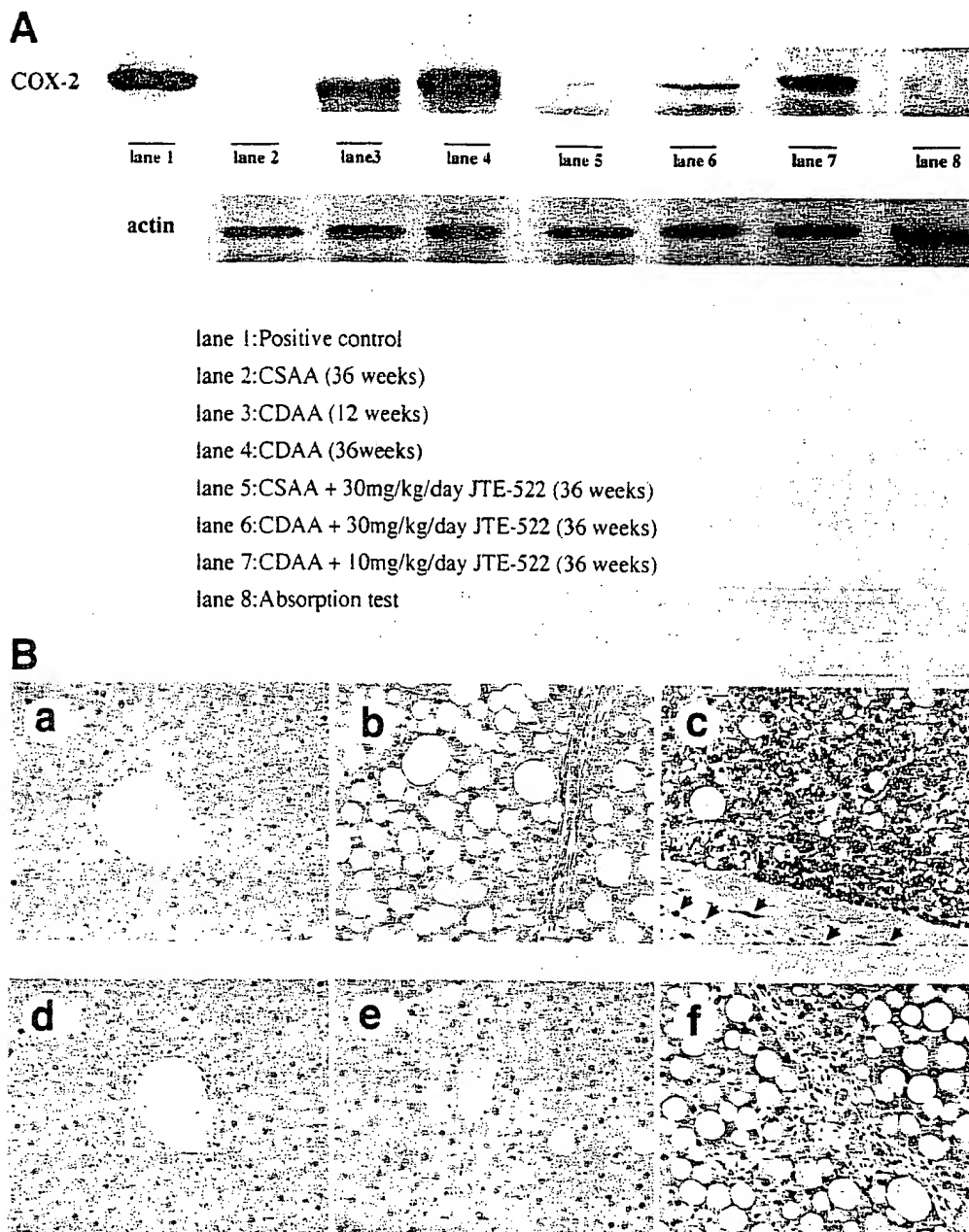
* P<0.05
** P<0.01
*** P<0.001

TAA-Induced Liver Fibrosis Model

Compared with normal control liver (n = 5), all TAA-treated rats (n = 8) developed marked liver fibrosis with pseudo-lobule formation in the whole liver. TAA-treated rats administered JTE-522 at 15 mg · kg^{-1} · day^{-1} (n = 8) generally showed a weak fibrogenic response in the liver, in which the normal liver architecture was largely well preserved. Collagen staining showed the extent of fibrogenesis in each group (Figure 11A), and the difference between the TAA group and the TAA plus JTE-522 group was significant (Figure 11B; P < 0.001). Western blot analysis showed that α-SMA largely increased in TAA-treated fibrotic livers and administration of JTE-522 reduced α-SMA expression (Figure 11C).

Discussion

Previous studies showed that the nonselective COX inhibitors piroxicam and acetylsalicylic acid prevented cirrhosis and development of preneoplastic nodules in CDAA-treated rats.[18,33] One of the aims of our study was to examine whether selective inhibition of COX-2 alone would be sufficient to realize this goal because such inhibition appears to yield much less toxicity than other nonsteroidal anti-inflammatory drugs. In experiment B, long-term administration of CSAA plus JTE-522 at 30 mg · kg^{-1} · day^{-1} did not induce any toxic side effects, such as body weight loss, gastrointestinal ulceration, bleeding, or renal toxicity, which are

A

lane 1:Positive control

lane 2:CSAA (36 weeks)

lane 3:CDAA (12 weeks)

lane 4:CDAA (36weeks)

lane 5:CSAA + 30mg/kg/day JTE-522 (36 weeks)

lane 6:CDAA + 30mg/kg/day JTE-522 (36 weeks)

lane 7:CDAA + 10mg/kg/day JTE-522 (36 weeks)

lane 8:Absorption test

B

Figure 6. COX-2 expression in rat livers. (A) Western blot analysis. *Upper panel, lane 1*: positive control of the lysate from a colon cancer tissue; *lane 2*: CSAA (36 weeks); *lane 3*: CDAA (12 weeks); *lane 4*: CDAA (36 weeks); *lane 5*: CSAA containing 30 mg · kg^{-1} · day^{-1} JTE-522 (36 weeks); *lane 6*: CDAA containing 30 mg · kg^{-1} · day^{-1} JTE-522 (36 weeks); *lane 7*: CDAA containing 10 mg · kg^{-1} · day^{-1} JTE-522 (36 weeks); *lane 8*: absorption test. Preabsorbed antibody yielded no band in the lysate from CDAA-treated liver for 36 weeks. *Lower panel*: blots for actin serve as loading controls. (B) Immunohistochemistry. (a) Normal liver (CSAA, 36 weeks). (b) Fibrous liver (CDAA, 12 weeks). (c) Cirrhotic liver (CDAA, 36 weeks). Hepatocytes expressed COX-2 protein in the cytoplasm and perinuclear region. Note that mononuclear cells and fibroblast-like cells in fibrous tissues also expressed COX-2 (*arrows*). (d) CSAA plus high-dose JTE-522, CDAA plus (e) high- or (f) low-dose JTE-522 for 36 weeks. (Original magnification 400×.)

major problems caused by nonsteroidal anti-inflammatory drugs.[34] Other investigators also reported that a selective COX-2 inhibitor, SC-236, did not impair renal function in a rat model with liver cirrhosis[35] and that a COX-2 selective inhibitor, celecoxib, did not cause damage of the gastroduodenal mucosa.[36]

CDAA-induced early events include fibrosis, formation of preneoplastic lesions, fatty changes, and loss of hepatic lobule architecture. The results of experiment A (experiments on early events) clearly showed that JTE-522 caused about a 69% and 95% decrease of fibrogenesis when used at low and high doses, respectively (Table 2). The longer experiment B showed a further increase in CDAA-associated fibrogenesis, and the extent of inhibition accomplished by JTE-522 was approximately proportional to that achieved in experiment A. These out-
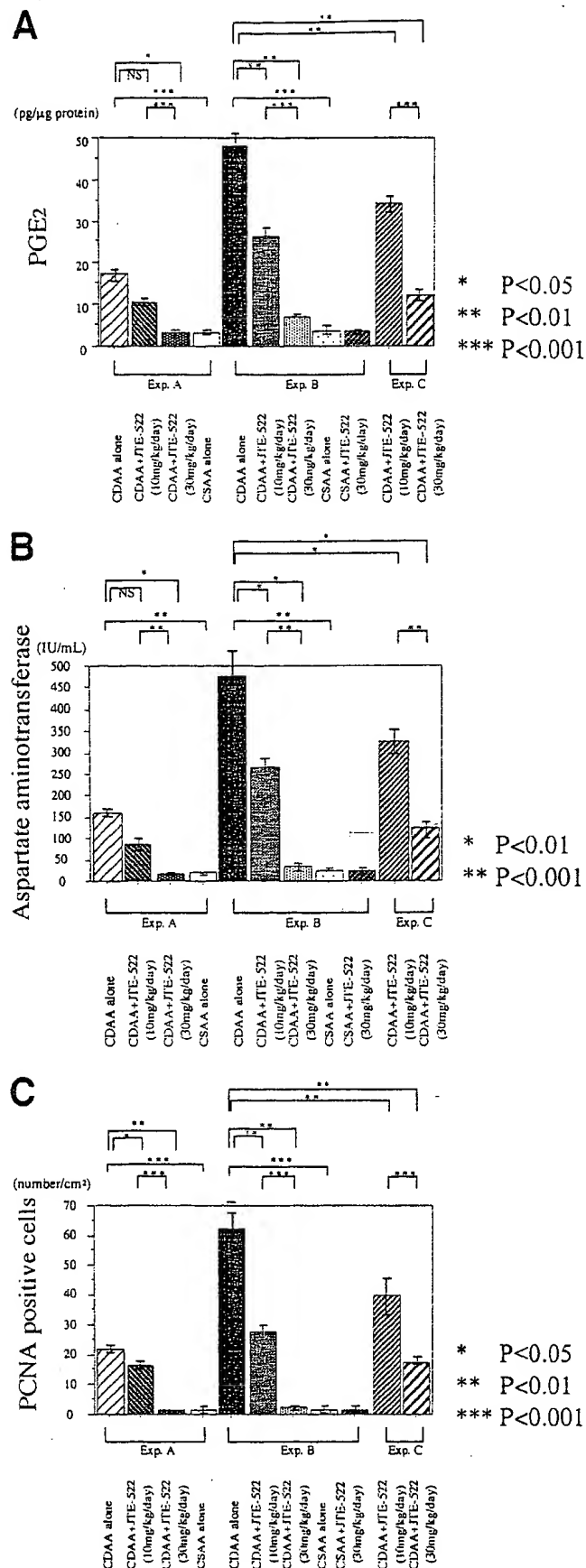
Figure 7. (A) PGE$_2$ levels. (B) serum aspartate aminotransferase levels, and (C) PCNA expression in the liver. Each parameter was examined in all rats in experiments A–C. Aspartate aminotransferase level was closely associated with that of PGE$_2$. Data are mean ± SD.
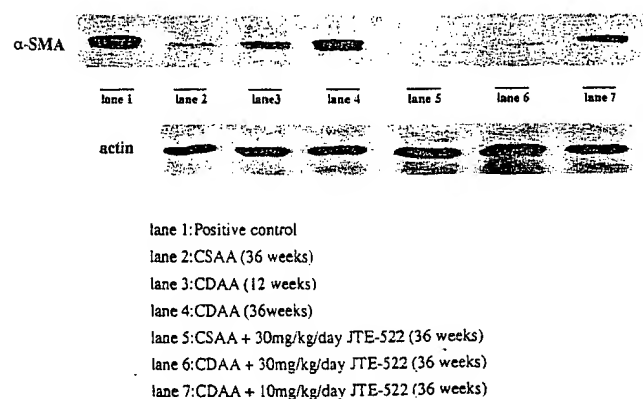
standing results strongly suggest that JTE-522 may be a potent agent against CDAA-induced liver fibrosis. CDAA resulted in the appearance of preneoplastic foci as early as 12 weeks, and development of the lesions was time dependent. The number and area of GST-P–positive foci reflect the potential of initiation and promotion of the preneoplastic lesions, respectively.[22,37] JTE-522 inhibited both processes in a dose-dependent manner.

With regard to the long-term effects of CDAA, high frequencies of cirrhosis and HCC were noted at 36 weeks. Cirrhosis is the final stage of liver fibrosis, and JTE-522 eventually inhibited the development of cirrhosis even when used at a low dose. At the end of experiment B, we were surprised to find that CDAA plus JTE-522–treated rats had normal liver appearance although the livers of most CDAA-treated rats contained multiple small nodules consisting of cirrhotic nodules and HCCs. Detailed microscopic examination confirmed the absence of cirrhotic nodules and HCCs in JTE-522–treated groups. Moreover, even though JTE-522 was administrated from 12 weeks (experiment C), no cirrhosis or HCC were noted at 36 weeks.

Other investigators have also provided sufficient evidence from animal studies indicating that selective COX-2 inhibitors produce effective prevention of carcinogenesis induced by various reagents. However, in these in vivo studies, the suppression rate for cancer formation was only partial even with the highest experimental dose. For example, nimesulide inhibited generation of carcinomas of the bladder, breast, and tongue by 60%, 20%, and 74%, respectively.[38–40] Celecoxib inhibited development of carcinoma of the colon and bladder both by 57%.[41,42] JTE-522 inhibited development of esophageal squamous cell carcinoma by 62%.[17] On the other hand, we found that HCC was very common among CDAA-treated rats (13 of 15; 85%), whereas it was completely inhibited in all CDAA plus JTE-522–treated rats (0 of 10; 0%), that is, an inhibition rate of 100%. Moreover, although JTE-522 exerted a clear dose-dependent inhibition of liver fibrosis and preneoplastic lesions, it did not show dose dependency on cancer formation. These findings may reflect the unique process of hepatocarcinogenesis. Thus, it is probable that establishment of cirrhotic liver may be a prerequisite for further development of HCC in the CDAA model. We postulate that inhibition of fibrogenesis by JTE-522 prevented the development of cirrhosis with effective protection against hepatocarcinogenesis. Because livers showed early changes associated with fibrosis and preneoplastic lesions during the long experiments, even in JTE-522–treated groups, we cannot exclude the possible development of HCC with
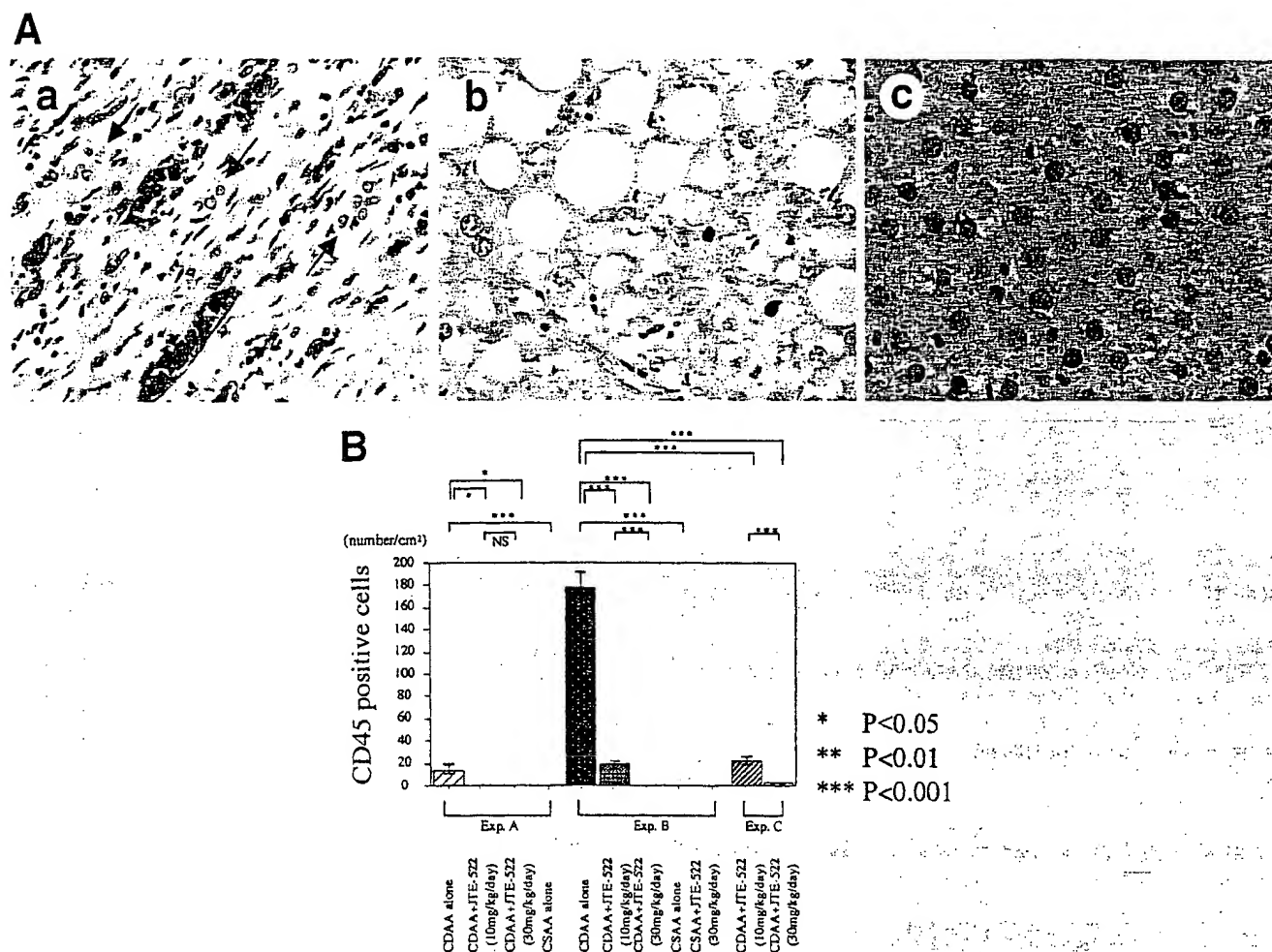
Figure 8. (*A*) Immunostaining for CD45 expression in the rat liver. (*a*) CDAA-treated rat liver for 36 weeks. CD45-positive inflammatory cells often appeared. *Arrows* indicate duct-like structure of hepatocytes and no inflammatory cells. Jurkat cells served as positive control (data not shown). (*b*) CDAA plus 10 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} JTE-522 for 36 weeks. (*c*) CDAA plus 30 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} JTE-522 for 36 weeks. (Original magnification 400×.) *B*) Density of CD45-positive cells determined in all rats in experiments A–C. Data are mean ± SD.

lane 1: Positive control
lane 2: CSAA (36 weeks)
lane 3: CDAA (12 weeks)
lane 4: CDAA (36 weeks)
lane 5: CSAA + 30mg/kg/day JTE-522 (36 weeks)
lane 6: CDAA + 30mg/kg/day JTE-522 (36 weeks)
lane 7: CDAA + 10mg/kg/day JTE-522 (36 weeks)

Figure 9. Western blot for α-SMA expression in liver tissue. *Upper panel, lane 1*: positive control of human cirrhotic liver tissue; *lane 2*: CSAA (36 weeks); *lane 3*: CDAA (12 weeks); *lane 4*: CDAA (36 weeks); *lane 5*: CSAA containing 30 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} JTE-522 (36 weeks); *lane 6*: CDAA containing 30 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} JTE-522 (36 weeks); *lane 7*: CDAA containing 10 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} JTE-522 (36 weeks). *Lower panel*: blots for actin served as loading controls.

further extension of the duration of the experiments. With regard to cancer development, it is notable that c-myc level was highly up-regulated in rat livers of the CDAA group, which is consistent with the previous report.[43] Interestingly, JTE-522 significantly decreased c-myc expression at a low dose and especially at a high dose, at least at the messenger RNA level, although the precise mechanism of HCC formation is not certain at present.

Western blot and immunohistochemistry showed that COX-2 was up-regulated from fibrous liver to cirrhosis in CDAA-treated groups. The mechanism(s) of COX-2 up-regulation is not known. However, it is postulated that transforming growth factor α is up-regulated in CDAA-treated rat liver and this molecule can induce COX-2 expression.[44,45] Alternatively, inflammation-associated cytokines such as tumor necrosis factor α and interleukin 1β can also induce COX-2.[46,47] By contrast, treatment with JTE-522 clearly decreased COX-2 expression dose dependently and the extent of liver fibrosis
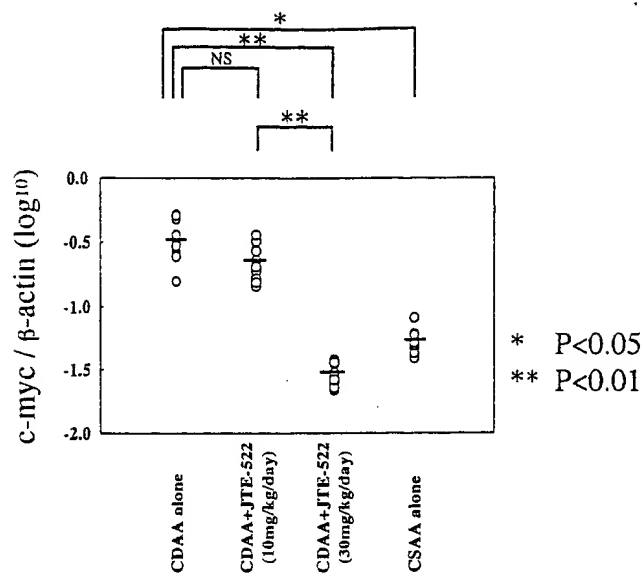
Figure 10. Quantitative reverse-transcription PCR for c-myc messenger RNA. c-myc messenger RNA levels in livers of rats fed CDAA for 36 weeks, CDAA plus low-dose JTE-522, CDAA plus high-dose JTE-522, and CSAA were measured by Light Cycler after reverse transcription. c-myc expression was normalized with rat β-actin expression. CDAA induced a high level of c-myc expression, and JTE-522 at a high dose significantly inhibited induction of c-myc gene expression.

was closely associated with COX-2 level (Figure 6B). JTE-522–induced reduction of PGE_2 levels in the CDAA group ensured efficacy of the agent in vivo and suggests that COX-2–mediated arachidonate metabolism may play an essential role in the progression of rat fibrosis. However, we cannot exclude the possible involvement of targets other than COX-2 in this process.

CDAA-induced formation of preneoplastic lesions is mediated by a repeated sequence of liver cell necrosis and regenerative cell proliferation and probably involves oxidative DNA damage.[18,19] We found that the pathologic changes of liver correlated well with the extent of oxidative DNA damage as assessed by levels of 8-hydroxydeoxyguanosine (data not shown).[48] It appears that JTE-522 neutralized oxidative DNA damage and decreased transaminase levels through reduction of PGE_2 production. Although the precise mechanism of action of JTE-522 on the prevention of CDAA-induced fibrogenesis and hepatocarcinogenesis is not certain at present, our study provided several clues on the mechanism.

In both human and rat hepatocarcinogenesis, fibrosis is a major process that increases gradually from normal liver to cirrhosis. Activated hepatic stellate cells (also
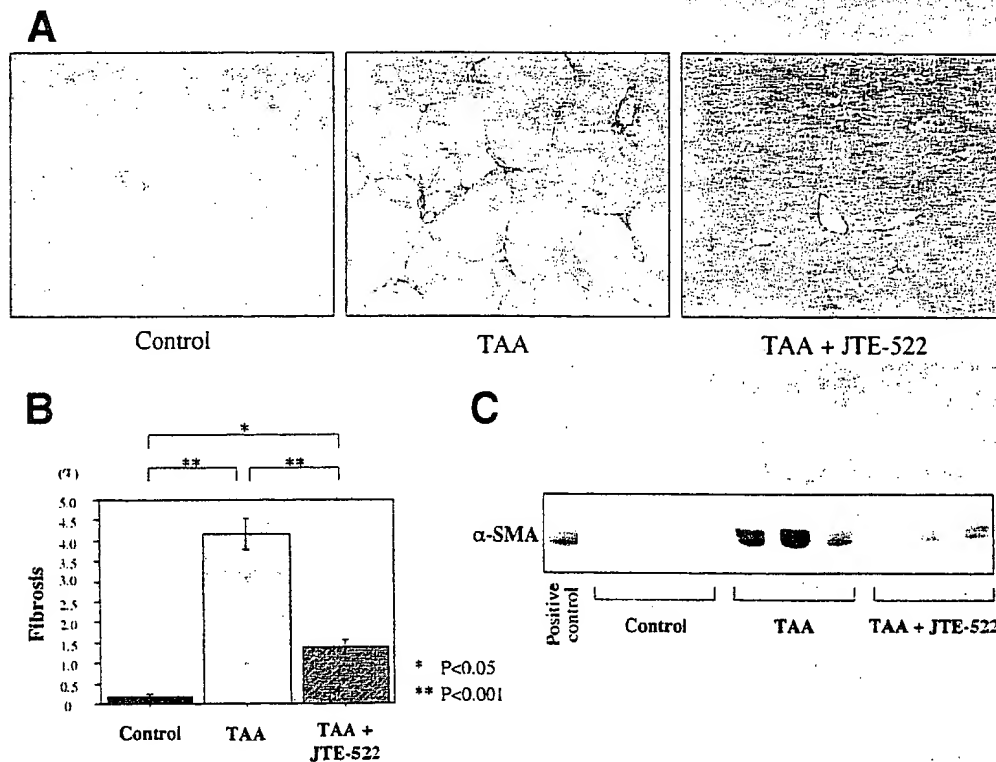
Figure 11. TAA-induced liver fibrosis model. (*A*) Collagen staining. TAA-treated rats showed marked liver fibrosis with pseudo-lobule formation, whereas TAA-treated rats administered JTE-522 (15 mg · kg⁻¹ · day⁻¹) show only weak fibrotic process in the liver. (*B*) Quantification of collagen staining. The difference between the TAA group and the TAA plus JTE-522 group was significant (*P* < 0.001). (*C*) Western blot analysis for α-SMA. TAA-treated fibrotic livers showed marked expression of α-SMA, and JTE-522 reduced the expression. Positive control: human cirrhotic liver tissue. Three representative samples for each group are shown. Equal loading of the proteins was confirmed by Ponso-S protein staining of the membrane (data not shown).

designated as fat-storing cells) are believed to play a crucial role in liver fibrogenesis, and collagen is the major component.[49] α-SMA is expressed by activated hepatic stellate cells but not by quiescent hepatic stellate cells; therefore, α-SMA serves as an appropriate marker for activated hepatic stellate cells.[20,32] Our results showed up-regulation of α-SMA from normal liver to fibrotic liver to cirrhosis and efficient suppression of α-SMA by JTE-522 in the CDAA group. The data suggest that JTE-522 may inhibit activation of hepatic stellate cells and lead to prevention of fibrogenesis. In vitro assay using hepatic stellate cell cultures may clarify this hypothesis.

Several studies indicate that COX-2 inhibitors suppress cell growth.[50,51] We previously found that JTE-522 had potent growth-suppressive effects on cholangiocellular carcinoma cells.[9] Therefore, it is assumed that this growth-suppressive effect of JTE-522 might lead to relatively slow regeneration of the proliferative hepatocytes, allowing a gradual process of fibrogenesis. We indeed found a low proliferative activity in JTE-522–treated livers as assessed by PCNA staining. Alternatively, choline deficiency is known to induce hepatocyte apoptosis in vitro and in vivo.[52] Although inhibition of COX-2 can induce apoptosis in several cell culture systems, recent studies indicate that the COX-2 inhibitor rather blocks apoptotic pathway in certain situations.[53,54] This possibility should be also considered.

Another possibility is that the anti-inflammatory effects of JTE-522 might contribute to repression of persistent inflammation and ultimately lead to a reduction of fibrogenesis. It is known that persistent inflammation promotes human liver fibrogenesis, as often seen in hepatitis C virus–infected liver.[55] By histologic examination of livers in the CDAA group, we found that infiltrative inflammatory cells appeared in liver fibrosis and cirrhotic livers had quite severe inflammation. This finding was further confirmed by CD45 staining that could detect all types of leukocytes. It should be emphasized here that CDAA-induced liver fibrosis in rats is substantially different from hepatitis C virus–based fibrosis with respect to the underlying pathologic process (e.g., oxidative DNA damage vs. viral infection). However, the present findings of PCNA expression, activated hepatic stellate cells, and liver inflammation in our rat CDAA model are of potential importance because dysregulation of these parameters is also seen in human liver fibrosis.[32,49,55,56]

We focused on the effect and mechanism of JTE-522–mediated inhibition of CDAA-induced liver fibrosis, but does this compound inhibit liver fibrogenesis in a mechanistically distinct form of liver disease? To address this

question, we performed additional experiments using the TAA-induced liver fibrosis model. Our results showed that, even with a moderate dose ($15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$), JTE-522 also inhibited liver fibrosis in TAA-treated rats when estimated by collagen staining and α-SMA expression. Although the underlined mechanism of the protective effect of JTE-522 against TAA-induced fibrosis should be explored in the next stage, the results at least indicate that JTE-522–mediated inhibition of liver fibrosis is rather a universal effect and is not limited to the CDAA model alone.

In conclusion, the present study suggests that COX-2 may be the principal enzyme involved in the development of rat experimental liver fibrosis and that JTE-522 is an effective chemopreventive agent with low toxicity.

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